UNIVERSITI PUTRA MALAYSIA

PARTIAL PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE S-TRANSFERASE FROM THE LIVERS OF MALAYSIAN CATFISH (CLARIAS BATRACHUS) AND MALAYSIAN RED TILAPIA (OREOCHROMIS SP.)

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By

CHIN CHEE MENG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Dedicated to my parents

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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July 2005

Chairman : Professor Nor Aripin Shamaan, PhD

Faculty : Biotechnology and Biomolecular Sciences

The present study is concerned mainly with partial purification and characterization of cytosolic GST from livers of Malaysian catfish and Malaysian red tilapia as both species are of economic and commercial importance in Malaysia being the major freshwater fishes consumed. This study hopes to establish the patterns of cytosolic glutathione S-transferase isoenzymes in the catfish and red tilapia. This may be useful to further achieve an understanding toward this enzyme in view of using it as a tool in environmental monitoring. The hepatic GST enzyme from catfish and red tilapia was partially purified 15X and 27X respectively in comparison to the ultra-centrifuged cytosolic fraction by affinity chromatography. Specific GST activity of 12.69 unit/mg protein and 33.42 unit/mg protein was obtained from livers of catfish and red tilapia using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Isolation of GST isoenzymes from affinity purified fractions was achieved by preparative isoelectric-focusing. Two isoenzymes; one major isoenzyme designated Ci1 and one minor isoenzyme

designated Ci2 were isolated from catfish liver with an apparent pI of 6.20 and 8.73 respectively. One isoenzyme designated Ti1 was isolated from red tilapia liver with an apparent pI of 9.14. SDS-PAGE analysis suggests that the isolated isoenzymes appear to be homodimeric in nature with subunit molecular weight of 29.7 ±1.7 kDa (Ci1), 27.7 ±1.3 kDa (Ci2) and 29.9 ±0.9 kDa (Ti1). As has been found for most GSTs, highest catalytic activity was obtained with CDNB. With the exception of the isoenzyme from cytosol and affinity purified fractions of red tilapia, none of the catfish fractions displayed enzyme activities towards 1,2-dichloro-4-nitrobenzene (DCNB) and ethacrynic acid (EA). Therefore, both catfish and tilapia possess hepatic glutathione S-transferase activity, indicating that they are capable of conjugating endogenous or xenobiotic metabolites/compounds as a result of foreign exposure or oxidative metabolism with glutathione, thereby making it a useful tool as a effective biomarker of aquatic contamination.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PURIFIKASI SEPARA DAN PENCIRIAN ENZIM GLUTATHIONE S-TRANSFERASE DARIPADA HATI IKAN KELI MALAYSIA (*CLARIAS BATRACHUS*) DAN IKAN TILAPIA MERAH MALAYSIA (*OREOCHROMIS SP.*)

Oleh

CHIN CHEE MENG

July 2005

Pengerusi : Profesor Nor Aripin Shamaan, PhD

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Kajian ini mengenai purifikasi separa dan pencirian GST sitosol daripada ikan keli Malaysia dan ikan tilapia merah Malaysia disebabkan kedua-dua spesis tersebut mempunyai kepentingan ekonomi dan komersial di Malaysia memandangkan ia adalah ikan air tawar yang sering dimakan. Kajian ini diharapkan dapat menentukan corak isoenzim sitosol GST dalam hati ikan keli dan tilapia merah. Ini mungkin berguna untuk mencapai pemahaman ke atas enzim ini dimana ia boleh dijadikan sebagai alat untuk memantau persekitaran. Enzim GST hepatik daripada ikan keli dan tilapia merah telah disepara-tulen sebanyak 15X dan 27X masing-masing dibandingkan dengan fraksi sitosol ultra-centrifuged oleh kromatografi afiniti. Aktiviti spesifik enzim yang diperolehi adalah 12.69 unit/mg dan 33.42 unit/mg daripada hati ikan keli dan tilapia merah masing-masing dengan menggunakan 1-chloro-2,4-dinitrobenzene (CDNB) sebagai substrat. Pemencilan isoenzim GST daripada fraksi yang ditulenkan dengan kromatografi afiniti dicapai dengan menggunakan "preparative

isoelectric-focusing". Dua isoenzim; satu isoenzim major Ci1 dan satu isoenzim minor Ci2 dipencilkan daripada hati ikan keli dengan nilai pI 6.20 dan 8.73 masing-masing. Satu isoenzim Ti1 dipencilkan daripada hati ikan tilapia merah dengan nilai pI 9.14. Analisis SDS-PAGE mencadangkan isoenzim-isoenzim yang dipencilkan mempunyai struktur homodimer dengan berat molekul subunit 29.7 ±1.7 kDa (Ci1), 27.7 ±1.3 kDa (Ci2) dan 29.9 ±0.9 kDa (Ti1). Seperti yang ditemui dengan kebanyakan GST yang lain, aktiviti katalitik yang tertinggi diperolehi dengan CDNB. Dengan pengecualian isoenzim daripada sitosol dan fraksi yang ditulen oleh kromatografi afiniti daripada ikan tilapia merah, tiada fraksi ikan keli yang menunjukkan aktiviti dengan 1,2-dichloro-4-nitrobenzene (DCNB) dan ethacrynic acid (EA). Oleh itu, kedua-dua ikan keli dan tilapia mempunyai aktiviti glutathione S-transferase, menunjukkan bahawa mereka mampu mengkonjugasikan metabolik/bahan endogenus atau xenobiotik akibat daripada terdedah kepada persekitaran atau metabolisma oksidatif dengan glutathione, oleh itu membuatkannya alat yang berguna sebagai petanda biologi untuk pencemaran akuatik.

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I certify that an Examination Committee met on 20th July 2005 to conduct the final examination of Chin Chee Meng on his Master of Science thesis entitled "Partial Purification and Characterization of Glutathione S-Transferase from the Liver of Malaysian Catfish (*Clarias batrachus*) and Malaysian Red Tilapia (*Oreochromis* sp.)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows :

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

CHIN CHEE MENG

Date : 12/8/2005

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α	alpha
β	beta
Br	bromine
BSA	bovine serum albumin
CDNB	1-chloro-2,4-dinitrobenzene
Cl	chlorine
C-terminal	carboxyl-terminal
Cys	cysteine
Δε	molar extinction coefficient
DCNB	1,2-dichloro-4-nitrobenzene
DDT	dichlorodiphenyltrichloroethane
DNA	deoxyribonucleic acid
ε	epsilon
E.C.	Enzyme Commission
EA	ethacrynic acid
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
GDP	gross domestic product
Glu	glutamate
Gly	glycine
GS^-	glutathione thiolate ion
GSH	reduced glutathione

G-site	glutathione binding site
GST	glutathione S-transferase
HPLC	high performance liquid chromatography
H-site	hydrophobic binding site
I	iodine
KCl	potassium chloride
kDa	kilo Dalton
M	molar
MAPEG	Membrane Associated Proteins in Eicosanoid and Glutathione metabolism
mol	mole
MPOB	Malaysian Palm Oil Board
mRNA	messenger RNA
MW	molecular weight
NaCl	natrium chloride
N-terminal	amino-terminal
θ	theta
ρ	rho
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
-SH	sulfurhydryl group
π	pi
TEMED	N, N, N', N'-tetramethylethylenediamine

μ	mu
U	unit
UPM	Universiti Putra Malaysia
γ	gamma

CHAPTER I

INTRODUCTION

The Malaysian fisheries sector plays an important role in providing food and as a source of protein to the general public. In 2000, it contributed about 1.60% to the national Gross Domestic Product (GDP)[a] and provided direct employment to 82,000 fishermen and 22,000 fish culturists. With the recent episodes of Nipah virus infecting pigs and the bird flu attack in chickens, more people are turning to other sources of proteins such as fish. Therefore, the Department of Fisheries is taking several measures in order to provide a sustained production of fish in the country. Although deep sea fishing still remains as the easiest method for fish production, however water pollution and over-fishing are taking its toll on the fish population in the wild causing shortages and resulting in the increasing prices of fish. Therefore, the real potential lies in the aquaculture industry in order to achieve a sustainable growth in fish production.

Freshwater aquaculture contributes a significant percentage (30.2%) to the total aquaculture production in 2000. Some of the major freshwater species cultured were freshwater Malaysian Catfish (*Clarius batrachus*) and Red Tilapia (*Oreochromis sp.*).

[a] National GDP RM 339.42 billion (Statistics Handbook, Statistics Department Malaysia, 2001

The catfish are demersal creatures with the ability to survive in muddy and polluted water. Generally, they are found in rivers and swampy areas. They are omnivorous in nature surviving on water insects, worms, shrimps, snails and rotting materials such as plants. They have a special breathing system which allows them to survive for short periods of time on land. In Malaysia the Malaysian catfish is usually mated with the African catfish (*Clarias gariepinus*) as the African catfish displays higher growth rate and can attain a size of 1 kg in 6 months. The red tilapia can be found in freshwater, saltwater and also brackish water bodies. They are also omnivorous, surviving on a diet of worms, plants and small fish (Basir and Daud, 1994).

As the two species represent an economic importance in Malaysia, maximum yield during the breeding and production are of utmost importance to maximize profits. Therefore, there is a need for a monitoring system to monitor the state of the fish habitat and the stress levels of the fish (i.e overcrowding, parasite infections).

Glutathione S-Transferases (GSTs, E.C. 2.5.1.18) are a multigene family of multifunctional enzymes which can be found in bacteria, yeasts, higher plants, mollusks, crustaceans, insects, fish, amphibians and mammals (Jakoby, 1978; Mannervik, 1985b). They catalyze the nucleophilic attack of the sulfur atom of glutathione on compounds possessing an electrophilic center (Hayes and Pulford, 1995). Therefore, GSTs play a key role in the cellular detoxification process in by conjugating a broad spectrum of endobiotic and xenobiotic electrophilic substrates (as a result of

environmental exposure or oxidative metabolism) with intracellular glutathione into more readily excretable products (Mannervik and Danielson, 1988; Hayes and Pulford, 1995; Armstrong, 1997).

Certain GST isoenzymes display selective response towards pollutants and high activity towards certain substrates, making it a useful tool as an effective biomarker of aquatic contamination (Egaas et al., 1999; Gallagher et al., 1996).

Objectives

The objectives of the present study are to:-

1. Partially purified the cytosolic glutathione S-transferase (E.C. 2.5.1.18) from the livers of Malaysian catfish (*Clarius batrachus*) and red tilapia (*Oreochromis sp.*)
2. Partially characterized the cytosolic glutathione S-transferase from the livers of Malaysian catfish (*Clarius batrachus*) and red tilapia (*Oreochromis sp.*)

Ultimately, this study hopes to establish the patterns of the cytosolic glutathione S-transferases (i.e. the dimeric structures, specific activities, pI values, molecular weight and substrate specificity) in the catfish and red tilapia. This may be useful to further achieve an understanding toward this enzyme in view of using it as a tool in environmental monitoring.

CHAPTER II

LITERATURE REVIEW

The Enzymes of Detoxification

All living organism are continuously subjected to foreign external (xenobiotics) compounds; naturally occurring or man-made such as chemicals in the air, water, food additions and drugs or endogenous toxicants produced as a result of the metabolism of hormones, steroids and oxidative metabolism. Most of these compounds are lipophilic and might be excreted slowly from the body. Therefore, the toxicity of the compounds is increased as a result of the accumulation in the tissues of the organism. Given these exposures, the ability to detoxify is important to the overall health and the survivability of the organism.

The process of detoxification through which the non-polar (lipophilic) toxins are converted to polar (hydrophilic) compounds that can be readily excreted by the organism occurs in two classical steps known as Phase 1 and Phase 2. Each of the phases involved the so-called detoxification enzymes that have broad specificity towards a wide variety of toxins faced by the organism. The two phases are interconnected; Phase 1 reactions normally generate intermediate end-products that are toxic to the tissues. Phase 2 reactions conjugates the end-products of Phase 1 reactions to yield final products that are eliminated from the organism. Phase 1 reactions are characterized by the cytochrome P450 supergene family of isoenzymes that catalyzed the addition

of a reactive group to the substrate (toxins). However the product yields a compound that is more toxic compared to the parent compound. Furthermore, the reactions of Phase 1 enzymes generate very reactive damaging free radicals. Therefore it is necessary that this compound is further metabolized by the Phase 2 enzymes to avoid damage of the organism's proteins and genes. Phase 2 reactions consist of conjugating enzymes that normally adds a water-soluble molecule to the active site of the Phase 1 metabolite to facilitate its removal in urine or bile. These conjugates are usually less toxic and more water-soluble than their precursors. However, not all toxins undergo detoxification using the Phase 1 to Phase 2 route. Occasionally the parent toxins undergo detoxification directly through the Phase 2 route bypassing the Phase 1 route. Of all the Phase 2 enzymes, glutathione S-transferases (GSTs E.C. 2.5.1.18), the most abundant protein in the cytosolic fraction of the liver (Booth *et al.*, 1961; Wilce and Parker, 1994), is the most widely studied and major Phase 2 detoxification enzyme catalyzing the conjugation of cellular nucleophile glutathione with a wide range of endogenous or xenobiotic hydrophobic molecules (Armstrong, 1997; Hayes and Pulford, 1995; Mannervik and Danielson, 1988).

Glutathione

Reduced glutathione (GSH), a tripeptide consisting of γ-glutamyl-cysteinyl-glycine can be conjugated in the initial step of mercapturic acid synthesis (George, 1994). Its cysteinyl residue provides a nucleophilic thiol important for the detoxication of electrophilic metabolites. Its net negative charge and overall hydrophilicity increases the aqueous solubility of the lipophilic